

# ArfGAP3 Regulates the Transport of Cation-Independent Mannose 6-Phosphate Receptor in the Post-Golgi Compartment

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## Summary

ArfGAPs are known to be involved in cargo sorting in COPI transport. However, the role of ArfGAPs in post-Golgi membrane traffic has not been defined. To determine the function of ArfGAPs in post-Golgi traffic, we used small interfering RNA to examine each of 25 ArfGAPs for effects on cation-independent mannose 6-phosphate receptor (CIMPR) localization. We found that downregulation of ArfGAP3 resulted in the peripheral localization of CIMPR. The effect was specific for ArfGAP3 and dependent on its GAP activity, because the phenotype was rescued by ArfGAP3 but not by ArfGAP1, ArfGAP2, or the GAP domain mutants of ArfGAP3. ArfGAP3 localized to the *trans*-Golgi network and early endosomes. In cells with reduced expression of ArfGAP3, Cathepsin D maturation was slowed and its secretion was accelerated. Also retrograde transport from the endosomes to the *trans*-Golgi network of endogenous CIMPR, but not truncated CIMPR lacking the luminal domain, was perturbed in cells with reduced expression of ArfGAP3. Furthermore the exit of epidermal growth factor receptor (EGFR) from the early endosomes and degradation of EGFR after EGF stimulation was slowed in cells with reduced expression of ArfGAP3. ArfGAP3 associates with Golgi-localized,  $\gamma$ -ear-containing, ADP-ribosylation factor binding proteins (GGAs), and ArfGAP3 knockdown reduces membrane association of GGAs. A possible mechanism explaining our results is that ArfGAP3 regulates transport from early endosomes to late endosomes. We suggest a model in which ArfGAP3 regulates Golgi association of GGA clathrin adaptors.

## Results and Discussion

### ArfGAP3 Depletion Changed the Distribution of CIMPR

To screen for ArfGAPs that function in post-Golgi traffic, we treated HeLa cells with small interfering RNA (siRNA) targeting 25 ArfGAPs and examined the localization of cation-independent mannose 6-phosphate receptor (CIMPR) (see [Supplemental Information](#) available online). In cells transfected with ArfGAP3 siRNA, CIMPR was dispersed in the periphery ([Figure 1B](#)) as compared to control cells in which CIMPR concentrated in the juxtanuclear region. We confirmed that siRNA-treated cells (henceforth called ArfGAP3 knockdown cells) had reduced levels of ArfGAP3 protein by western blot ([Figure 1A](#)) and immunofluorescence ([Figure 1B](#)).

ArfGAP3 is reported to be involved in COPI transport in the *cis*-Golgi [1–4], whereas our results indicated function of ArfGAP3 in post-Golgi trafficking. For a more detailed localization of ArfGAP3, we double stained cells with antibodies against ArfGAP3 and the *trans*-Golgi network (TGN) marker TGN46, the medial-Golgi marker giantin, or the *cis*-Golgi marker GM130. ArfGAP3 colocalized with TGN46 to a greater extent than with GM130 ([Figures 1C and 1D](#)). HA-tagged Rab4, GFP-tagged Rab5, and RFP-tagged Rab7 were overexpressed as markers for early/recycling, early, and early/late endosomes. ArfGAP3 colocalized the most with Rab5, less with Rab7 and Rab4 ([Figures 1E, 1F, S1A, and S1B](#)). Thus, ArfGAP3 predominantly localizes at the TGN and early endosomes, consistent with a function of ArfGAP3 in post-Golgi trafficking.

To rule out a general disruption of Golgi function in ArfGAP3 knockdown cells, we measured the transport of a temperature-sensitive mutant of Vesicular Stomatitis Virus G protein (VSVG) to the cell surface ([Figure 1G](#)). We used cells treated with Brefeldin A (BFA), which disrupts the Golgi, as a positive control. In ArfGAP3 knockdown cells, transport was decreased only 12% compared to control cells. The distribution of Golgi marker proteins was not perturbed ([Figures 1H, 2H, 2J, S2A, and S2C](#)). Thus, the Golgi was largely intact and functional.

We quantified CIMPR distribution ([Figure 1H](#); see [Supplemental Information](#)). CIMPR localized to the periphery in 71% of ArfGAP3 knockdown cells in contrast to 22% of control cells ([Figure 1I](#)). Overexpression of ArfGAP3 rescued CIMPR dispersion due to ArfGAP3 knockdown ([Figures 1J and 1K](#)), excluding an off-target effect of the siRNA. ArfGAP3 is reported to function in ER-Golgi transport redundantly with ArfGAP1 and 2 [3]. However, CIMPR distribution in ArfGAP1 and ArfGAP2 knockdown cells was not different from in control cells ([Figure 1I](#)), and dispersion was not rescued by myc-tagged ArfGAP1 or HA-tagged ArfGAP2 ([Figure 1K](#)). These results indicate that the effect on CIMPR distribution was specific for ArfGAP3.

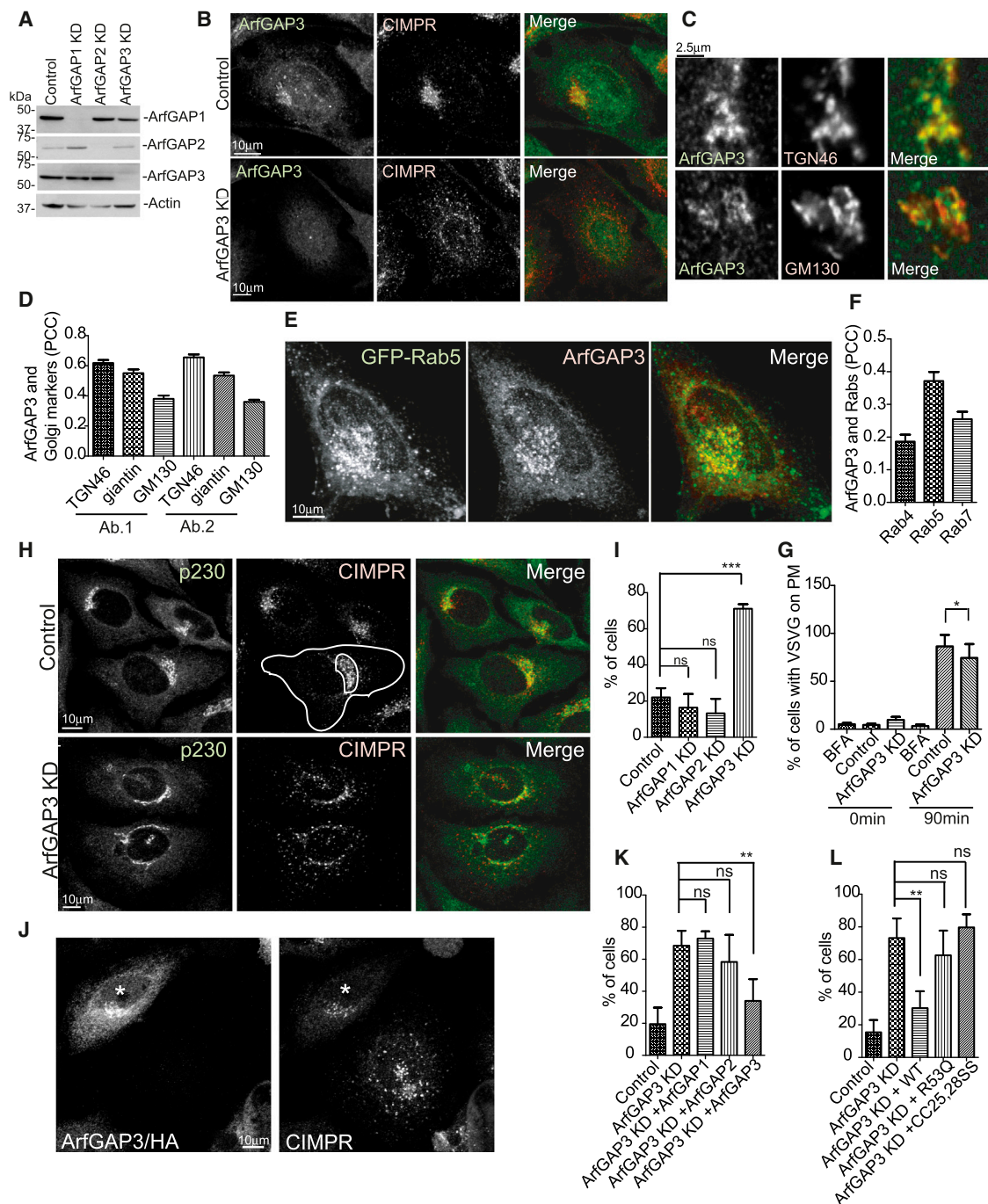
To determine whether ArfGAP3 GAP activity is required for CIMPR localization, we used two GAP-dead mutants, [R53Q]ArfGAP3 and [CC25,28SS]ArfGAP3. [R53Q]ArfGAP3 has a mutation in the catalytic arginine [5]. [CC25, 28SS]ArfGAP3 has double mutations in the GAP domain and is unable to bind to Arf-GTP and has no GAP activity. As shown in [Figure 1L](#), neither mutant rescued the effect of ArfGAP3 knockdown. The result indicates that GAP activity is required for ArfGAP3 function.

### Reduced Expression of ArfGAP3 Perturbs the Transport of CIMPR

To determine whether function of CIMPR is affected by ArfGAP3 knockdown, we examined processing and secretion of a ligand of CIMPR, the lysosomal enzyme Cathepsin D [6]. In pulse-chase experiments, we observed an increase of secreted Cathepsin D in the medium and a decrease of the mature form in ArfGAP3 knockdown cell lysates ([Figures 2A–2C](#)).

To determine the compartment to which CIMPR was relocated in ArfGAP3 knockdown cells, we analyzed its

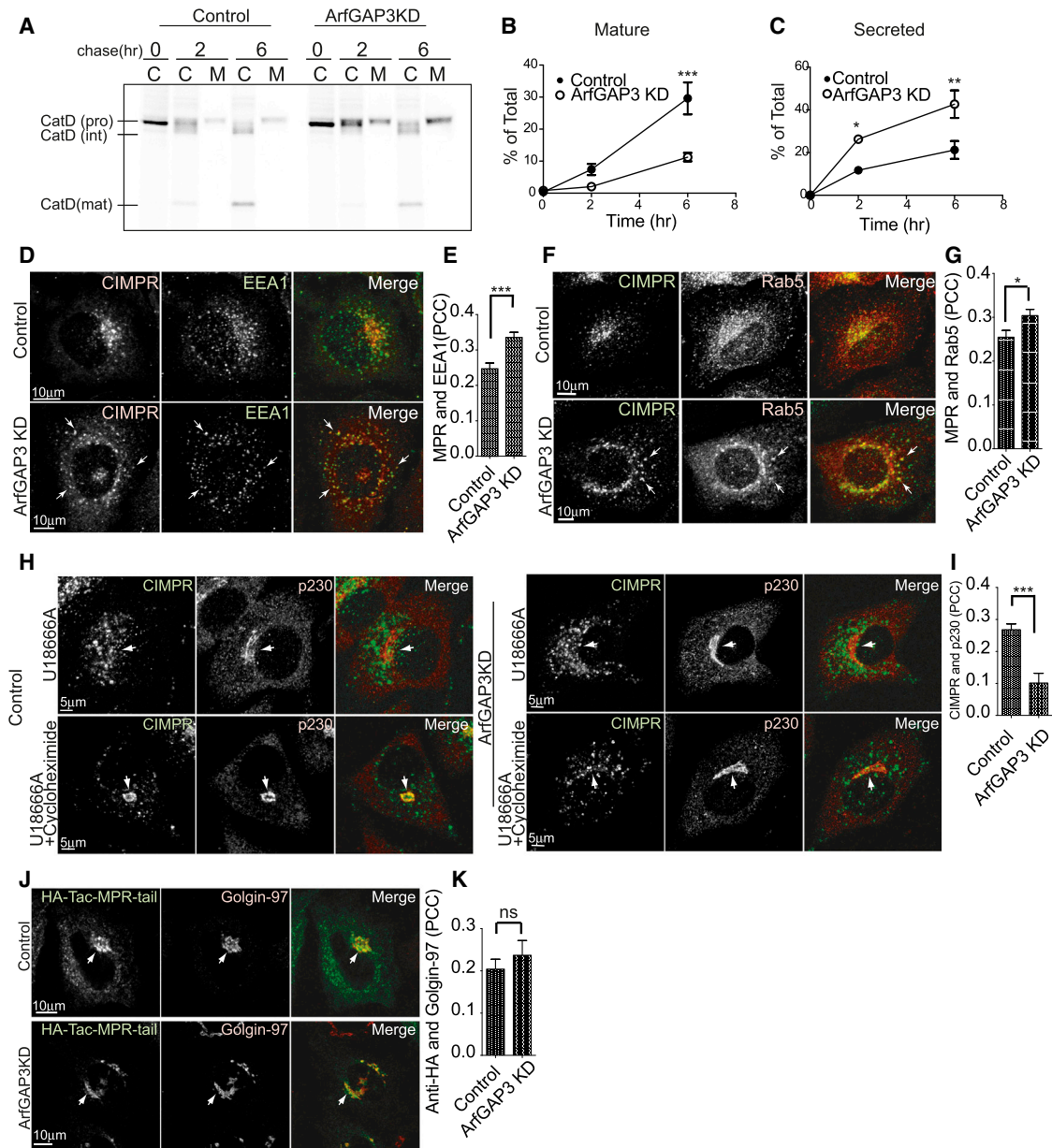
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**Figure 1. CIMPR Was Redistributed to the Periphery in ArfGAP3 Knockdown Cells**

(A) HeLa cells were transfected with control, ArfGAP1, 2, or 3 siRNAs. The cell lysates (30  $\mu$ g) were examined by immunoblotting.  
 (B) HeLa cells treated with either control or ArfGAP3 siRNA were double stained with ArfGAP3 and CIMPR antibodies.  
 (C) HeLa cells were double stained with anti-ArfGAP3 antibody and TGN46 or GM130.  
 (D) Z-stacks of images were analyzed for colocalization of ArfGAP3 with TGN46, giantin, and GM130. The average Pearson's correlation coefficients (PCCs) (n = 30) are presented with SEM. Two different antibodies against ArfGAP3 were used (Ab.1 and Ab.2).  
 (E) HeLa cells were transfected with GFP-Rab5 and stained with anti-ArfGAP3.  
 (F) Quantification of colocalization between ArfGAP3 and overexpressed Rabs (Rab4, Rab5, and Rab7). The average PCCs (n = 30) are presented with SEM.  
 (G) VSVG ts045 transport to the cell surface was measured by flow cytometry.  
 (H) Control HeLa cells and ArfGAP3 knockdown (KD) cells were double stained with CIMPR and p230. Total and Golgi area intensity of fluorescence were measured as shown by the outline.  
 (I) The percentage of cells with dispersed CIMPR was quantified for control and knockdown cells.  
 (J) Cells overexpressing HA-tagged ArfGAP3, indicated with the asterisk, retained a juxtanuclear localization of CIMPR.  
 (K) The effect of ArfGAP3 depletion on CIMPR distribution was reversed by ArfGAP3 but not ArfGAP1 or ArfGAP2 overexpression.  
 (L) GAP activity of ArfGAP3 was required for CIMPR localization.

\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001. ns, not significant. See also [Figure S1](#).



**Figure 2. CIMPR Transport Is Perturbed in ArfGAP3 KD Cells**

(A) Cells were pulse-labeled with [<sup>35</sup>S] methionine/cysteine for 1 hr, chased for 2 or 6 hr, and then cell-associated and medium-associated proteins were immunoprecipitated with anti-Cathepsin D antibody. A representative experiment out of three is shown. The lanes indicated C are the cell-associated and lanes labeled M are medium-associated radiolabeled Cathepsin D.

(B) Mature Cathepsin D was decreased in ArfGAP3 KD cells. The amount of mature Cathepsin D detected in the autoradiograms presented in (A) was quantified.

(C) Quantification of pro-Cathepsin D detected in the cell-culture media. More Cathepsin D was secreted in ArfGAP3 KD cells.

(D) Control and ArfGAP3 KD cells were double stained with CIMPR and EEA1 antibodies. Arrows indicate colocalization of CIMPR with EEA1.

(E) Quantification of colocalization between CIMPR and EEA1. The average PCCs (n = 30) are presented with SEM.

(F) Control and ArfGAP3 KD cells were double stained with CIMPR and Rab5 antibodies. Arrows indicate colocalization of CIMPR with Rab5.

(G) Quantification of colocalization between CIMPR and Rab5. The average PCCs (n = 30) are presented with SEM.

(H) Endosome to TGN transport of endogenous CIMPR is slowed in ArfGAP3 knockdown cells. Cells were treated with 3  $\mu$ g/ml U18666A for 36 hr, thereby trapping CIMPR in endosomes. Cells were subsequently treated with 40  $\mu$ g/ml cycloheximide for 3 hr, which results in the transport of CIMPR to the TGN in control cells but not in ArfGAP3 KD cells. The cells were stained with CIMPR and p230. Arrows indicate the Golgi area stained with p230.

(I) Quantification of colocalization between endogenous CIMPR and p230 in cells treated with U18666A and cycloheximide. The average PCCs (n = 30) are presented with SEM.

(J) ArfGAP3 does not affect CIMPR lacking luminal domain. HeLa cells overexpressing HA-Tac-MPR-tail were incubated with anti-HA antibody for 3 min, washed, and chased for 30 min. The cells were stained with Golgin-97. Arrows indicate HA-Tac-MPR-Tail localized at the Golgi.

(K) Quantification of colocalization between internalized anti-HA antibody and Golgin-97. The average PCCs (n = 30) are presented with SEM.

\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; ns, not significant. See also [Figure S2](#).



colocalization with the early endosomal markers, EEA1 and Rab5. More CIMPR colocalized with EEA1 and Rab5 in ArfGAP3 knockdown cells than in controls (Figures 2D–2G). Colocalization with the recycling endosome marker, transferrin (Figures S1C and S1D), and the late endosome markers CD63 and Rab9 was unchanged (Figures S3A–S3D). These results indicate that CIMPR relocated in part to early endosomes in ArfGAP3 knockdown cells.

Endocytosis and recycling of transferrin were unchanged (Figures S1E and S1F), suggesting constitutive endocytosis and recycling pathways were not perturbed in ArfGAP3 knockdown cells.

To determine whether retrograde transport from the endosome to the Golgi was affected in ArfGAP3 knockdown cells, we examined the transport of a construct that has the cytoplasmic tail of CIMPR conjugated with HA-tagged interleukin 2 receptor luminal and transmembrane regions (HA-Tac-MPR-tail). We internalized anti-HA antibody to follow the transport of HA-Tac-MPR-tail from the plasma membrane (PM) to the Golgi. In 30 min, anti-HA antibody reached the Golgi and colocalized with Golgin-97, a TGN marker similarly in control and ArfGAP3 knockdown cells (Figures 2J and 2K). We also examined the retrograde transport of Shiga toxin B subunit, the cytoplasmic tail of Furin conjugated with CD8, and the cytoplasmic tail of sortilin conjugated with HA-Tac and found no differences between control and ArfGAP3 knockdown cells (Figures S2A–S2C). These results suggest that retrograde transport is not generally affected by ArfGAP3 depletion. We found that trafficking of the truncated MPR-tail differs from that of the full-length CIMPR (Figures S2D and S2E) consistent with previous studies [7]. We analyzed the retrograde transport of GFP-MPR-full by internalizing anti-GFP antibody for 30 min (Figure S2F). Internalized anti-GFP colocalized with TGN46 less in ArfGAP3 knockdown cells than in control cells (Figures S2F–S2H). Due to the low efficiency of transport of internalized GFP-MPR-full to the Golgi in control cells (Figure S2G), we used U18666A, which causes the accumulation of cholesterol in late endosomes and redistributes CIMPR to endosomal structures [7–9]. This peripherally localized CIMPR is shifted to the TGN upon cycloheximide treatment [7]. CIMPR was redistributed to the periphery in control and ArfGAP3 knockdown cells in the presence of U18666A (Figure 2H). Upon treatment with cycloheximide, CIMPR was transported back to the TGN in control cells but remained in the periphery in ArfGAP3 knockdown cells (Figures 2H and 2I). These results indicate that the transport from the endosome to the TGN of full-length CIMPR is specifically perturbed in ArfGAP3 knockdown cells.

#### The Transport of Epidermal Growth Factor Receptor Was Also Perturbed in ArfGAP3 Knockdown Cells

We next determined if the degradation pathway was affected by ArfGAP3 knockdown. After confirming equivalent levels of steady-state CIMPR expression between control and ArfGAP3 knockdown cells (Figure S3E), we monitored CIMPR degradation after treatment with cycloheximide (Figure S3F). No difference was detected between control and ArfGAP3 knockdown cells (Figure S3F). However, CIMPR was only slowly degraded, with significant CIMPR remaining after 7.5 hr of cycloheximide treatment in control cells, making detection of a small effect on the degradation pathway difficult.

We next examined the degradation of epidermal growth factor receptor (EGFR). Upon EGF stimulation, EGFR is

efficiently transported to the lysosomes via early and late endosomes [10]. In ArfGAP3 knockdown cells, the degradation of EGFR was slower than in control cells (Figures 3A and 3B). This effect could be due to a decrease in lysosomal enzyme activity, or a block in transport of EGFR to late endosomes/lysosomes. EGF partially colocalized with the lysosomal marker Lamp1 after 30 min of internalization in control cells treated with the lysosomal inhibitor leupeptin, whereas colocalization was significantly reduced in leupeptin-treated ArfGAP3 knockdown cells (Figures 3C and 3D), indicating the transport of EGF to the late endosomes/lysosomes is perturbed in ArfGAP3 knockdown cells. Colocalization of EGF with the early endosome marker EEA1 was increased in ArfGAP3 knockdown cells (Figures 3E and 3F), indicating the transport of EGF out of early endosomes is slowed. We found no significant difference in the colocalization of the fluid-phase marker BSA with Lamp1 in control and ArfGAP3 knockdown cells (Figures S3G and S3H). We also assayed endosomal acidification with transferrin conjugated with fluorescein isothiocyanate and found via the null point method [11] that endosomal acidification was not perturbed in ArfGAP3 knockdown cells (data not shown). These results indicate that the effect of ArfGAP3 knockdown is specific for EGFR and lysosomal biogenesis is not significantly perturbed.

Our results indicate that ArfGAP3 functions at the TGN-endosome interface. We examined if ArfGAP3 associates with TGN/endosomal coat proteins. Clathrin adaptor AP-1 and Golgi-localized,  $\gamma$ -ear-containing, ADP-ribosylation factor binding proteins (GGAs) are known to regulate MPR transport [12–15]. Also, ArfGAP3 is thought to regulate COPI in the early secretory pathway [1–4] and possibly endosomal COPI [16–21]. First, to examine if AP-1 or COPI associates with ArfGAP3, we overexpressed HA-tagged ArfGAP3 in HEK293 cells, immunoprecipitated with anti-HA antibody, and blotted for an AP-1 or COPI subunit (Figures 4A and 4B). We could not detect the association of AP-1 with ArfGAP3 (Figure 4A), whereas we detected the association of COPI with ArfGAP3 (Figure 4B), consistent with a previous report [22]. Mammalian GGAs have three isoforms, GGA1, GGA2, and GGA3 [23, 24]. GGA3 has two splicing variants, a ubiquitously expressed short form (GGA3S) and a brain-specific long form (GGA3L) [25, 26]. The short form lacks the binding site for CIMPR [25]. To examine if GGAs associate with ArfGAP3, we double transfected cells with HA-tagged ArfGAP3 and FLAG-tagged GGA1, GGA2, GGA3S, or GGA3L and performed immunoprecipitation with anti-HA. We found that FLAG-tagged GGA1, GGA2, and GGA3S were coprecipitated with HA-tagged ArfGAP3 (Figures 4C–4E), whereas FLAG-tagged GGA3L was not (Figure 4F). We also performed immunoprecipitation with anti-FLAG and then blotted with anti-HA (Figures 4G–4J). In these experiments, HA-tagged ArfGAP3 was coprecipitated with all GGAs (Figures 4G–4J). We performed immunoprecipitation from untransfected cells with anti-GGA2 antibody (Figure 4K). ArfGAP3 precipitated with GGA2 (Figure 4K), indicating endogenous ArfGAP3 and GGA2 associate.

We next examined whether ArfGAP3 affected the association of coat proteins with the TGN/endosomes. ArfGAP3 is an ArfGAP that catalyzes GTP hydrolysis on Arf. We reasoned that if ArfGAP3 is downregulated in cells, Arf·GTP would accumulate, and this accumulation would increase the efficiency of recruitment of coat proteins to membrane. We examined membrane association of coat proteins using image-based

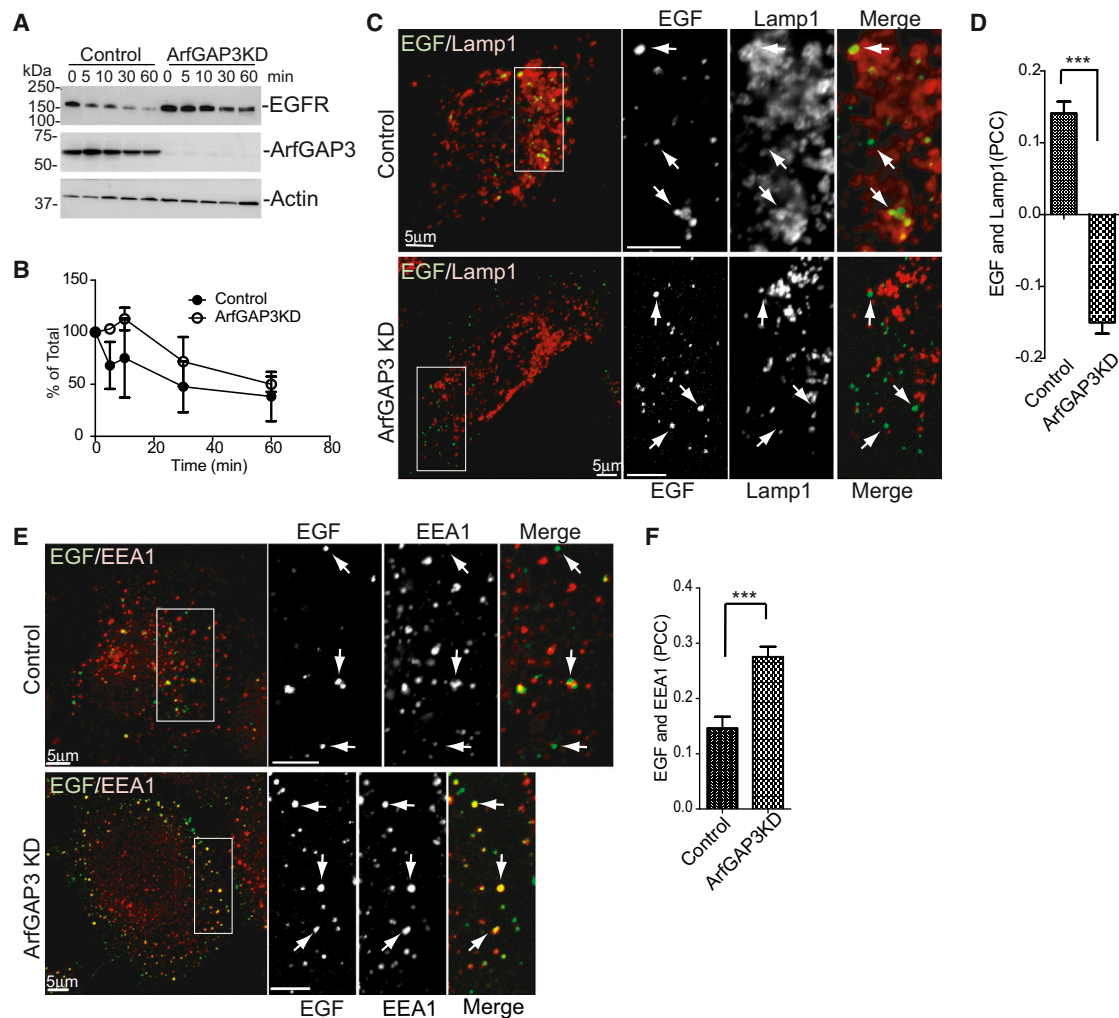


Figure 3. EGFR Transport Is Perturbed in ArfGAP3 KD Cells

(A) Effect of ArfGAP3 depletion on EGFR degradation. The cells were serum starved overnight then treated with EGF for the indicated times. EGFR levels were measured by immunoblotting. A representative experiment (out of three) is shown.

(B) Quantification of EGFR degradation in ArfGAP3-depleted cells. Immunoblots from three experiments were quantified by densitometry using ImageJ. The values at each time point were normalized to time 0. Average values  $\pm$  SEM are plotted.

(C) ArfGAP3 KD decreased targeting of EGF to the LAMP1 compartment. The cells were treated with 1 mg/ml leupeptin for 4 hr and then serum starved for 1 hr. Alexa-Fluor-488-conjugated EGF was internalized for 3 min, washed, and chased for 30 min. The cells were fixed and stained with Lamp1. Arrows indicate structures containing EGF.

(D) Quantification of colocalization between EGF and Lamp1 for experiments described in (C). The average PCCs ( $n = 30$ ) are presented with SEM.

(E) The cells were treated as in (B) and (C) and stained with EEA1. Arrows indicate structures containing EGF.

(F) Quantification of colocalization between EGF and EEA1. The average PCCs ( $n = 30$ ) are presented with SEM.

\*\*\* $p < 0.001$ . See also Figure S3.

analysis (see Supplemental Information; Figures S4A and S4B). We measured the ratio of Golgi/Cytosol for AP-1, COPI, GGA1, GGA2, GGA3, and Golgin-97 (Golgin-97 is reported to be recruited to the Golgi membrane by Arf-like protein, Arl1 [27]) (Figures 4L–4Q), in control and ArfGAP3 knockdown cells. We found no difference in Golgi association of COPI, AP-1, GGA3, and Golgin 97 (Figures 4L, 4M, 4P, and 4Q). In contrast, we found a small but significant decrease in Golgi/endosomal association of GGA1 and GGA2 (Figures 4N and 4O). Our results indicate that ArfGAP3 regulates GGA1/2.

We suggest that ArfGAP3 affects transport from early endosomes to late endosomes. First, CIMPR is partly relocated to early endosomes in ArfGAP3 knockdown cells (Figures

2D–2G). Second, ArfGAP3 affected EGFR transport from early to late endosomes (Figures 3E and 3F). Third, a significant pool of ArfGAP3 localized at early endosomes (Figures 1E and 1F). Fourth, ArfGAP3 affected transport of full-length CIMPR but not truncated CIMPR lacking the luminal region. Waguri et al. [7] proposed that the luminal region of CIMPR has a role in endosomal retention of the receptor. Full-length CIMPR is thought to be transported from early to late endosomes and then returns to the TGN [30], whereas truncated CIMPR is possibly transported from early endosomes to the TGN directly [31]. An effect of ArfGAP3 knockdown on full-length CIMPR and EGFR but not on truncated CIMPR is most easily explained if ArfGAP3 affects transport from early to late endosomes. We also considered the possibility that ArfGAP3

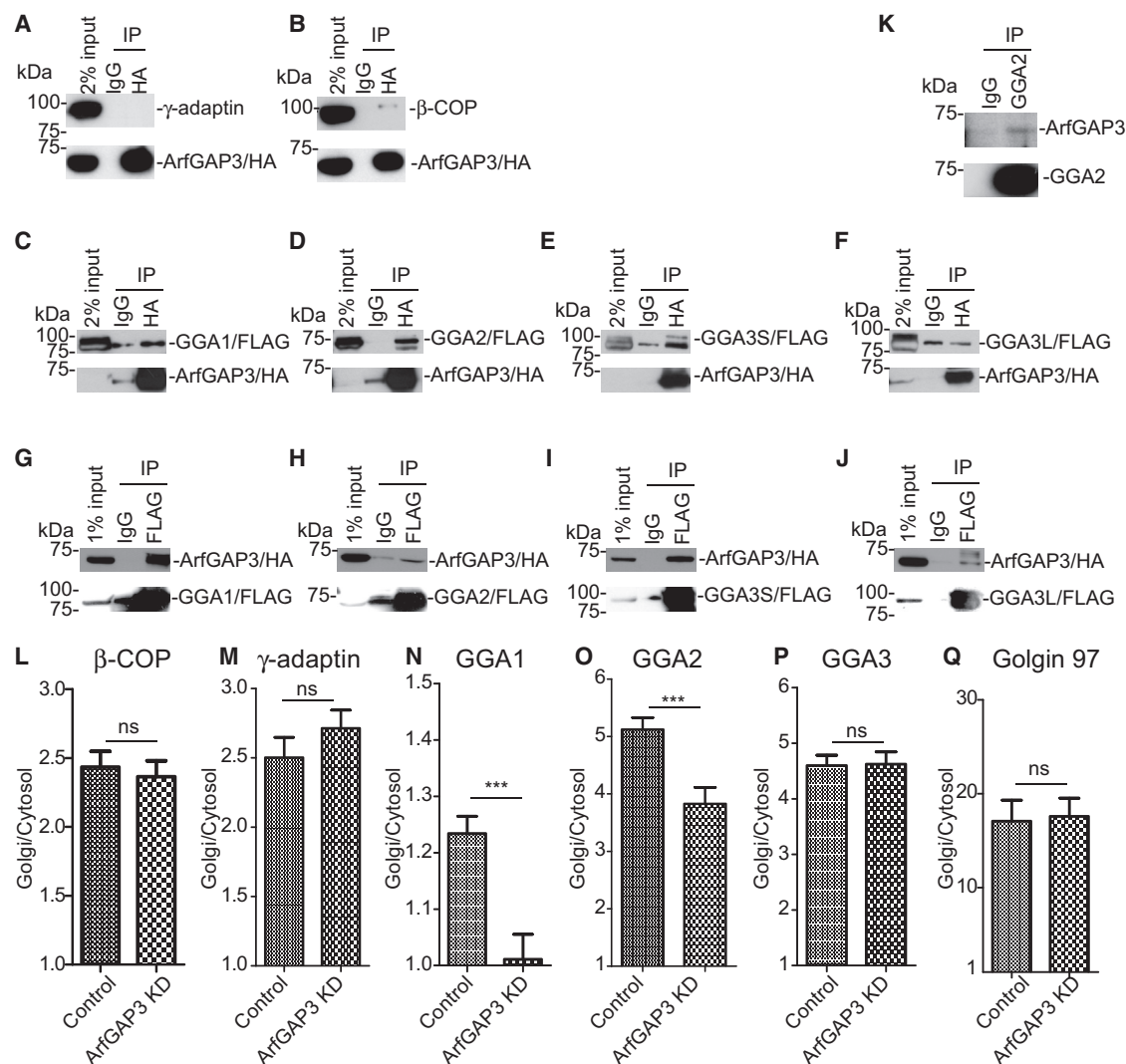


Figure 4. ArfGAP3 Associates with GGAs

(A and B) 293T cells were transfected with HA-tagged ArfGAP3 and immunoprecipitated with anti-HA and blotted with anti- $\gamma$ -adaptin (AP-1 subunit) (A) or anti- $\beta$ -COP (COPI subunit) (B). (C–F) 293T cells were double transfected with HA-tagged ArfGAP3 and FLAG-tagged GGA1 (C), GGA2 (D), GGA3S (E), GGA3L (F), immunoprecipitated with anti-HA and blotted with anti-FLAG antibody. (G–H) 293T cells were double transfected with HA-tagged ArfGAP3 and FLAG-tagged GGA1 (G), GGA2 (H), GGA3S (I), GGA3L (J), immunoprecipitated with anti-FLAG and blotted with anti-HA antibody. (K) Coimmunoprecipitation was performed with anti-GGA2 antibody and blotted with anti-ArfGAP3. A representative experiment (out of three) is shown. (L–Q) HeLa cells were stained with  $\beta$ -COP (L),  $\gamma$ -adaptin (M), GGA1 (N), GGA2 (O), GGA3 (P), and Golgin 97 (Q). Quantification of the ratio of Golgi/Cytosol was presented with SEM in control and ArfGAP3 KD cells.

\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns, not significant. See also Figure S4.

regulates CIMPR transport from late endosomes to the TGN, the step affected by knockdown of GCC188 and by RhoBTB3 [32–34]. However, in the case of knockdown of GCC188 or RhoBTB3, CIMPR colocalized with Rab9 but not EEA1. Our working hypothesis is summarized in Figure S4C.

We found that ArfGAP3 associates with GGAs and Golgi/endosomal association of GGA1 and GGA2 is decreased in ArfGAP3 knockdown cells. These findings are not predicted by the standard model in which Arf-GTP recruits coat and the GAP functions as a negative regulator of coat binding to membrane. However, evidence is accumulating that the ArfGAPs can have a positive function related to coat assembly

[28, 29, 35–37]. It is plausible that the decreased association of GGA1 and GGA2 with the Golgi/endosomal compartment on ArfGAP3 knockdown reflects ArfGAP3 functions as an Arf effector, interacting with GGA1/2 and driving the assembly of GGA1/2 to promote formation of vesicles, rather than functioning solely as a negative regulator of Arf.

In summary, we report the unexpected finding that ArfGAP3 functions nonredundantly in the post-Golgi compartment where it regulates transport of CIMPR and EGFR. Ongoing studies are focused on identifying binding partners of ArfGAP3 as well as other approaches for elucidating its role in cargo sorting.

## Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2013.07.087>.

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